Supporting Information

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Materials and Methods

Bacterial Membranes and Cytosolic Fraction Preparation. Bacteria were grown in liquid medium. Cells were harvested by centrifugation at 5,000 × *g* for 10 min. The bacterial pellet from 20 ml culture was washed with and resuspended in 2 ml TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA) and disrupted by ultrasonication on ice. Unbroken cells and debris were removed by centrifugation at 6,000 × *g* for 10 min. The supernatant was subjected to ultracentrifugation at 100,000 × *g* for 1 h. The cleared supernatant was retained as the cytosolic fraction and frozen at −80°C. The pellet, which is the total membrane fraction, was resuspended in 2 ml PBS and frozen at −80°C.

FISH Experiments: Tissue Samples. For each patient, we obtained samples from the resected ileum (2 × 2 cm) and biopsy samples of their neoterminal ileum 6 months later. Samples were processed directly in the operating room. Tissue samples were fixed in 4% formalin, incubated at 4°C for 48 h (72 h maximum), then processed and embedded into paraffin blocks according to routine procedures. Paraffin sections of 5 μm were placed on organosilane-coated slides and stored at room temperature. Biopsies were placed in Sarstedt 2.2-ml screw-cap tubes, immediately frozen in liquid nitrogen, and stored at −80°C until analysis.

Histological Examination. Sections were deparaffinized in xylene and rehydrated for 3 min in 100%, 96%, and 70% ethanol successively. They then were stained with hematoxylin and eosin for morphological assessment.

FISH. Before FISH, sections were deparaffinized, rehydrated, and postfixed in 4% paraformaldehyde for 5 min. Fixation was stopped after 5 min in PBS 3×, and slides were washed twice for 1 min in PBS 1×. Tissue sections were incubated 10 min at room temperature with Tris-EDTA buffer containing 10 mg/ml lysozyme and then washed using the hybridization solution (0.9 M NaCl, 20 mM Tris·HCl, pH 8, 0.01% SDS, and 30% formamide). Fixed tissue sections then were hybridized with the previous hybridization solution containing 4.5 ng/μl of 1 of the 5′-end Cy3-labeled 16S rRNA-targeted oligonucleotide probes. Hybridizations were performed at 35°C overnight in a microscope slide incubator, and stringent washings were carried out at 37°C (2 × 15 min) in a buffer containing 65 mM NaCl, 20 mM Tris·HCl, pH 8.0, 5 mM EDTA, and 0.01% SDS to remove nonspecific binding. The sections were mounted with Vectashield [mounting medium with 4′,6′ diamidino-2-phenylindole (DAPI); Vector Laboratories]. DNA was stained with DAPI to visualize all cells.

Quantification of Mucosa-Associated-Bacteria (MAB). Bacteria were visualized with an epifluorescence microscope Leica DMRB using Cy3- and DAPI-specific filters at 100×, 400×, and 1000× magnification, and images were captured with a Leica DFC 300 FX camera and FW 4000 software (Leica Microsystems SAS). The entire mucosal surface and epithelium of each 4-cm^2 tissue section was examined for the presence of bacteria. Pure cultured bacteria belonging to each group were hybridized as positive and negative controls for the FISH procedure. The quantification of total MAB for each tissue sample was performed when bacteria were detected with both the Eub338 probe and DAPI staining and without hybridization with the nonEub338 nonsense probe. The total number of bacteria was determined for each tissue sample and then divided by the total mucosal surface (mucosa length × thickness of paraffin sections). The proportion of bacteria hybridized with group-specific probes was expressed as a percent of total bacteria. Ten fields were examined for each sample, and the mean percentage was calculated.

PBMC Isolation and Stimulation. PBMCs were isolated from the peripheral blood of healthy donors as previously described (1). Briefly, after Ficoll gradient centrifugation (Amersham Pharmacia), mononuclear cells were collected, washed twice in RPMI medium 1640 (Live Technologies), and adjusted to 2 × 10^6 cells/ml in RPMI 1640 supplemented with gentamicin (150 μg/ml), 2 mM L-glutamine and 10% decomplemented FCS (Gibco-BRL) and seeded in 24-well tissue culture plates (Corning). Twenty microliters of a thawed bacterial suspension at 10^9 CFU/ml was added (bacteria:cell ratio of 10:1). PBS containing 20% glycerol was used as a negative (nonstimulated) control. After 24 h of stimulation at 37°C in an atmosphere of air with 5% CO_2, supernatants were collected, clarified by centrifugation, and stored at −20°C until cytokine analysis. Neither medium acidification nor bacterial proliferation was observed. Cytokines were measured by ELISA using BD PharMingen antibody pairs for IL-10, IL-12p70, and IFN-γ, according to the manufacturer's recommendations.

Experiments on Caco-2 Cell Lines. The human colon cancer cell line Caco-2 from the European Collection of Cell Cultures (Wiltshire, United Kingdom) was cultured in 12- to 24-wells culture plates in DMEM (Invitrogen) supplemented with 20% heat-inactivated FCS, 1% nonessential amino acids (Invitrogen) at 37°C in 10% CO_2/air atmosphere. Media were changed every day. Experiments were initiated on days 20–21 after seeding (previously shown as an early stage of differentiation, corresponding to the upper crypt–lower villus stage of differentiation). Twenty-four hours before bacterial challenge, the culture medium was changed for a medium without FCS. Bacteria were added at a multiplicity of infection (moi) of 100 in 0.5 ml DMEM. Cells were stimulated simultaneously with human recombinant IL-1β (15 μg/ml; Sigma–Aldrich) for 6 h. For some experiments, a challenge was made using bacterial supernatant at 40%, *F. prausnitzii* DNA, cytoplasm, or membrane in DMEM (bacterial supernatants were previously filtered through a 0.2-μm filter and added in DMEM). After incubation, cell supernatants were collected and frozen at −80°C.

IL-8 ELISA and Protein Concentration. Cells were washed twice with PBS and lysed in PBS containing 1% Triton X-100 and a protease inhibitor mixture (Roche Diagnostics GmbH). Protein concentrations were determined in cell lysates using bicinchoninic acid protein assay reagents (Pierce) according to the manufacturer's instructions. The IL-8 level was determined in cell supernatants using ELISA kit DuoSet (R&D Systems) according to the manufacturer's recommendations.

Construction of Stable NF-κB Reporting Cells. Stable transfectants of a Caco-2 cell line were obtained after transfection with the plasmid pNiFty2 (Invivogen). This plasmid combines five NF-κB sites with the SEAP reporter gene. Induction by NF-κB activates the promoter, resulting in the expression of the SEAP gene. Electroporation was performed using an amaxa nucleofector apparatus according to the manufacturer's instructions (Amaxa GmbH). After electroporation, cells were resuspended in complete fresh medium and seeded in 24-well plates for 96 h until

experiments. To establish stable clones, zeocin (50 μg/ml; Invitrogen) was added to the medium from 2 days after transfection for 10 days, and the medium was changed every 2 days. Resistant clones were isolated using cloning glass cylinders and tested by IL-8 secretion. Selected clones were further cultured in the presence of 25 μg/ml zeocin.

Analysis of NF-κB Activation. SEAP-transfected Caco-2 cells were cultured using the same protocol as for nontransfected Caco-2 cells, with 25 μg/ml zeocin in 12-well culture plates. Experiments were initiated on days 20–21 after seeding. Twenty-four hours before bacterial challenge, the culture medium was changed for a medium without FCS. Bacteria were added at an moi of 100 in 0.5 ml DMEM. Cells were simultaneously stimulated with IL-1β (15 μg/ml) for 6 h. After incubation, cell supernatants were collected for determination of secreted alkaline phosphatase. SEAP in the supernatant was revealed using Quanti-Blue (Invivogen). Absorbance at 655 nm was read after 3 h of revelation.

Mice. Seven- to eight-week-old conventional male BALB/c mice (Janvier) were used. All experiments were carried out in accordance with the institutional guidelines. Groups of 9 to 11 mice were used in each experiment. A total of 500 μl bacterial suspension, bacterial component solution, supernatant, or PBS was administered daily by intragastric gavage.

Cytokine Assay. Colon samples (segment of 0.5 cm located in the inflamed area) were mechanically homogenized in cold antiprotease buffer (Protease Inhibitor Mixture Tablets; Roche) using a miniBeadBeater (BioSpec Products), and after centrifugation for 30 min at 16,000 $\times$ *g* and 4°C, the supernatant was stored at −80°C. Total proteins were quantified in colon homogenates by a Bradford assay. Cytokines (TNF-α, IL-10, and IL-12) were quantified using ELISA kits (Ebioscience) according to manufacturer recommendations.

DNA Extraction from Fecal Samples. The distal colonic content from each mouse was collected after killing and aliquoted in Starstedt 2-ml screwcap tubes and frozen at −80°C. Total cellular DNA was extracted from 0.2 g fecal samples using the G'NOME kit (BIO101) modified by J.-P.F., *et al.*, unpublished results. The DNA extraction is described in the section on bacterial DNA extraction. DNA dissolved in 100 μl TE 1$\times$ buffer was stored at −20°C.

Design of Oligonucleotide Primers and Probes. The primers and probes for real-time quantitative PCR (qPCR) were designed using the Primer-Express software version 2.0 (Applied Biosystems). The selected primers and probe target sites (Table S2) were tested for specificity by submitting the sequences to the Probe Match program provided by Ribosomal Database Project II and on pure culture bacterial DNA (J.P.F., unpublished results). The primers were purchased from MWG-Biotech AG, and the TaqMan probes were synthesized by Applied Biosystems. The oligonucleotide probes used for FISH (2) were adapted to TaqMan probes as described by Furet *et al.* (unpublished data). The primers for this PCR were designed considering the RNA sequences (EMBL database) aligned with the program Clustal W. SYBR-Green qPCR was used to quantify subdominant species or group of species.

Real-Time qPCR. Real-time qPCR was performed by using an ABI 7000 Sequence Detection System apparatus with 7000 system software version 1.2.3 (Applied Biosystems). Amplification and detection were carried out in 96-well plates and with TaqMan universal PCR 2$\times$ master mix (Applied Biosystems) to quantify total bacteria and the bacterial-dominant group of microbiota, and with SYBR-Green PCR 2$\times$ master mix to the others. Each reaction was done in duplicate in a final volume of 25 μl with 0.2 μM final concentration of each primer, 0.25 μM final concentration of each probe, and 10 μl of appropriate dilutions of DNA samples. Amplifications were performed with the following temperature profiles: 1 cycle at 95°C for 10 min to denature DNA and activate AmpliTaq Gold polymerase, followed by 40 cycles of 95°C for 30 sec, 60°C for 1 min. For the SYBR-Green amplifications, the dissociation step was added to control the amplification specificity. We investigated the most important groups and species of the mouse fecal microbiota (Table S2).

Statistical Analysis. Statistical analysis for significant differences was performed according to the Student's *t*-test for unpaired data or by the nonparametric Mann–Whitney. differences with $P < 0.05$ were considered significant. Animal experiments were performed at least three times, and results from a representative experiment are shown.

1. Foligne B, *et al.* (2007) Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. *World J Gastroenterol* 13:236–243.
2. Lay C, *et al.* (2005) Design and validation of 16S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human faecal microbiota. *Environ Microbiol* 7:933–946.
3. Suzuki MT, Taylor LT, DeLong EF (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Appl Environ Microbiol* 66:4605–4614.
4. Huijsdens XW, *et al.* (2002) Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. *J Clin Microbiol* 40:4423–4427.

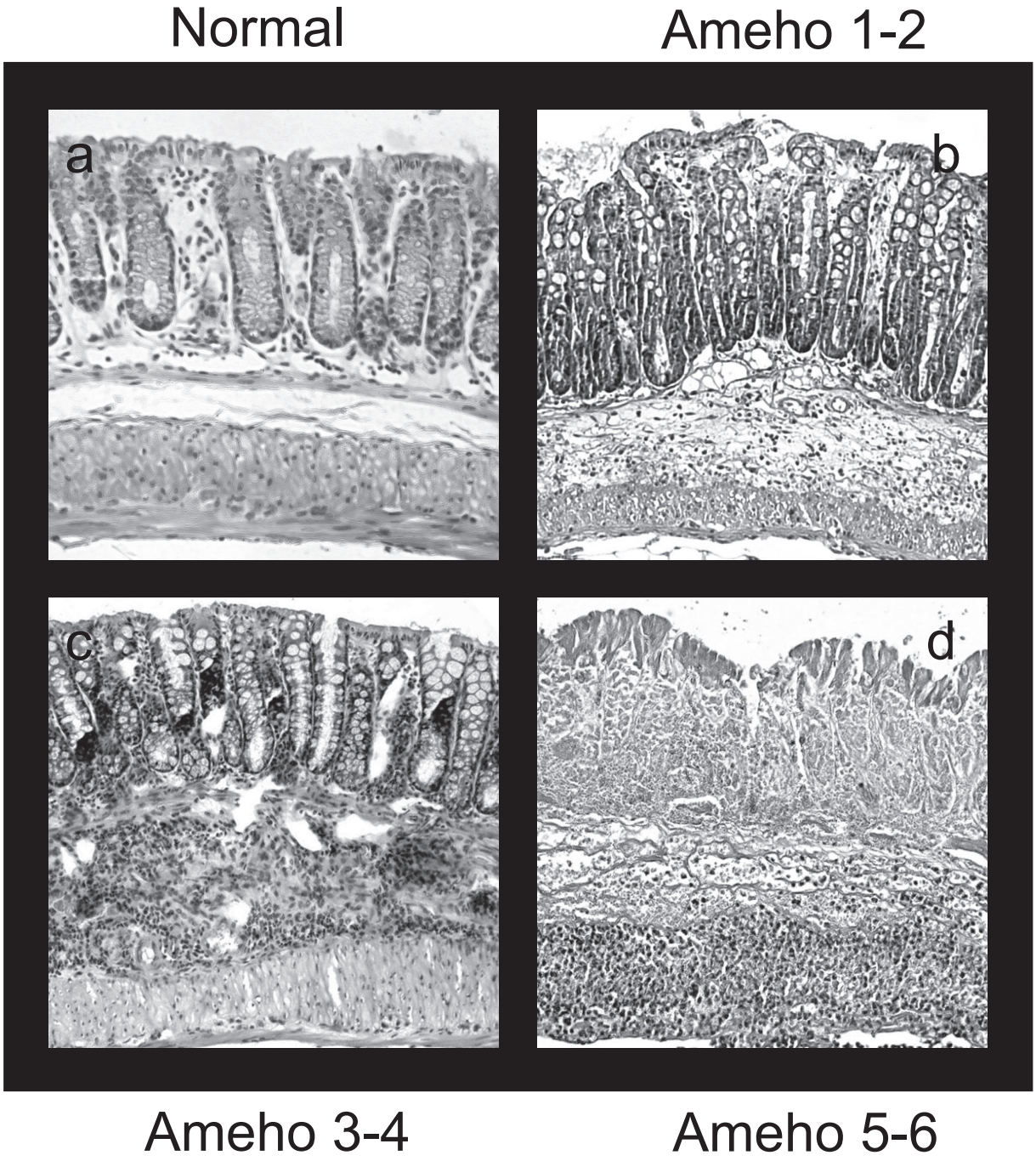

Fig. S1. Hematoxylin and eosin staining of representative cross-sections of murine (BALB/c) distal colon. (Magnification: ×20.) (*a*) Normal appearance of the colon from a negative control mouse. (*b*) Mild mucosal and submucosal inflammatory infiltrate (neutrophils) and edema. (*c*) Prominent inflammatory infiltrate (neutrophils) and edema, rare inflammatory cells invading the muscularis propriae without muscle necrosis. (*d*) Extensive coagulative necrosis bordered inferiorly by numerous neutrophils. Necrosis extends deeply into the muscularis propria.

Table S1. Composition of the ileal mucosa- associated microbiota (MAM) using fluorescent *in situ* hybridizationFISH (FISH) at the time of surgery and at 6 months after surgery. Assessment of the composition of the ileal MAM using FISH were performed using 6 group-specific probes (*Eubacteria*, *Bacteroides*, Enterics and relatives, *Bifidobacterium* genus, *Clostridium coccoides* and *Faecalibacterium prausnitzii*). The proportion of bacteria hybridized with group-specific probes was expressed as a percent of total bacteria. The Firmicutes proportion was obtained by adding *C. coccoides* and *F. prausnitzii* signals. MAM: Mucosa associated microbiota; * and § significant difference, *P*b = 0.02

	F. prausnitzii	*C. coccoides*	Firmicutes	Bacteroidetes	*Bifidobacteriaum*	Enterobacteria
MAM at time of surgery						
Endoscopic recurrence group	0.2 ± 0.2*	15.1 ± 8.4	15.4 ± 8.3	31.6 ± 12.2	3.6 ± 2.7	12.5 ± 8.0
No endoscopic recurrence group	3.3 ± 1.7*	16.3 ± 8.3	19.5 ± 8.5	32.0 ± 11.0	4.5 ± 2.4	1.5 ± 0.9
MAM at 6 months						
Endoscopic recurrence group	0.1 ± 0.1	14.6 ± 5.7	14.8 ± 5.7*§	31.3 ± 8.8	1.0 ± 0.7	9.3 ± 3.1
No endoscopic recurrence group	5.2 ± 3.8	30.0 ± 4.9	35.2 ± 5.0*§	50.3 ± 4.5	2.3 ± 1.2	3.5 ± 2.6

Assessment of the composition of the ileal MAM by using FISH was performed using six group-specific probes (Eubacteria, Bacteroides, Enterics, and relatives, *Bifidobacterium* genus, *C. coccoides*, and *F. prausnitzii*). The proportion of bacteria hybridized with group-specific probes was expressed as a percent of total bacteria. The Firmicutes proportion was obtained by adding *C. coccoides* and *F. prausnitzii* signals.
*Significant difference, $P = 0.02$

Table S2. Groups and species-specific 16S rRNA-targeted primers and probes used in this study. Probes are noted in bold.

Target		Primer and probe	Sequence 5′′- 3′′	Source of reference
Bacteria		F_Bact 1369	CGG TGA ATA CGT TCC CGG	Suzuki *et al.* (3)
		R_Prok1492	TAC GGC TAC CTT GTT ACG ACT T	
		P_TM1389F	**6FAM-CTT GTA CAC ACC GCC CGT C**	
C. leptum group	*C. leptum Ruminococcus albus, Ruminococcus flavefaciens F. prausnitzii*	Clept 09	CCT TCC GTG CCG SAG TTA	J.-P.F. *et al.*, unpublished work
		Clept 08	GAA TTA AAC CAC ATA CTC CAC TGC TT	
		P-Clep 01	**6FAM-CAC AAT AAG TAA TCC ACC**	
Bifidobacterium species	*Bifidobacterium*	F_Bifid 09c	CGG GTG AGT AAT GCG TGA CC	J.-P.F. *et al.*, unpublished work
		R_Bifid 06	TGA TAG GAC GCG ACC CCA	
		P_Bifid	**6FAM-CTC CTG GAA ACG GGT G**	
C. coccoides group	*C. coccoides*, *Eubacterium*, *Butyrivibrio*	Ccoc 07	GAC GCC GCG TGA AGG A	J.-P.F. *et al.*, unpublished work
		Ccoc14	AGC CCC AGC CTT TCA CAT C	
		P_Erec482	**VIC-CGG TAC CTG ACT AAG AAG**	
Bacteroides group	Bacteroides *Prevotella*	Bacter 11	CCT WCG ATG GAT AGG GGT T	J.-P.F. *et al.*, unpublished work
		Bacter 08	CAC GCT ACT TGG CTG GTT CAG	
		P_Bac303	**VIC-AAG GTC CCC CAC ATT G**	
Escherichia coli species		E.coli F	CAT GCC GCG TGT ATG AAG AA	Huijsdens *et al.* (4)
		E.coli R	CGG GTA ACG TCA ATG AGC AAA	
Faecalibacterium prausnitzii species	*F. prausnitzii*	Fprau 07 Fprau 02	CCA TGA ATT GCC TTC AAA ACT GTT GAG CCT CAG CGT CAG TTG GT	H.S. *et al.*, unpublished work
Lactobacillus		Lacto 05	AGC AGT AGG GAA TCT TCC A	J.-P.F.*et al.*,
Leuconostoc group		Lacto 04	CGC CAC TGG TGT TCY TCC ATA TA	unpublished work